REMARKS/ARGUMENTS

Claims 25 and 27-33 are pending. For convenience, the Examiner's rejections are addressed in the order presented in the October 18, 2004 Office Action. Although the present Office Action was clearly indicated to be non-final on the Office Action summary and at page 2, the Examiner indicated the Office Action was final at page 4. Given the confusion surrounding the status of the Office Action, Applicants respectfully request withdrawal of finality and entry and consideration of the Exhibits included herein. Applicants have filed a Notice of Appeal only to avoid abandonment of the application, should finality be maintained.

I. Rejections under 35 U.S.C. §101

Claims 25 and 27-33 are rejected under 35 U.S.C. §101 as allegedly lacking a specific and substantial utility. In response Applicants submit Exhibit A, a declaration from Dr. Yasumichi Hitoshi in support of the utility of Mkinase based on the information in the specification, *e.g.*, the cancer diagnostic use of the Mkinase nucleic acid, the ability of Mkinase to bind to TRAF4 and regulate signal transduction related to tumorigenesis, and the kinase activity associated with Mkinase. The declaration was first submitted in a related application, USSN 10/088,961, filed June 22, 2002, and directed to Mkinase proteins encoded by the Mkinase nucleic acids claimed herein.

Dr. Hitoshi asserts that the Mkinase protein is closely related to the NTKL protein disclosed in Kato *et al.* Kato *et al, Genomics* 79:760-767 (2002) is enclosed as Exhibit B; an alignment of the amino acid sequence of NTKL protein and the Mkinase protein is enclosed as Exhibit C. Dr. Hitoshi asserts that the alignment demonstrates that NTKL and Mkinase protein share 99% amino acid identity. He also states that Kato *et al.* demonstrate that the NTKL gene maps to a chromosomal breakpoint region on chromosome 11 that is associated with cancer. Thus, Dr. Hitoshi believes that more likely than not, the nucleic acid encoding Mkinase would be useful as a diagnostic for cancer.

Dr. Hitoshi also discusses the significance of the TRAF binding by the Mkinase protein. He points out that TRAF4 was recognized to have a role in tumorigenisis and that the

Mkinase protein binds to TRAF4 protein. Dr. Hitoshi asserts that based on the discovery of Mkinase associated kinase activity and the binding of the Mkinase to TRAF4, those of skill would believe that the Mkinase protein was involved in tumorigenesis. Finally, based on the relationship between Mkinase protein and the NTKL protein, the association between the NTKL gene (*i.e.*, the Mkinase gene) and a cancer-associated chromosomal breakpoint region, Mkinase associated kinase activity, and binding of the Mkinase to TRAF4, Dr. Hitoshi asserts that those of skill would have recognized the utility of the Mkinase protein, *i.e.*, the product of the claimed nucleic acid sequences.

The Office Action states that the asserted utility in the specification, *i.e.*, use of the claimed nucleic acids for diagnostic or prognostic information on cancer, is not a specific utility because allegedly cancer is not a specific disease. This is incorrect; cancer is properly designated as a specific disease. Applicants submit as Exhibit E a definition of cancer from page 505 of Harrison's Principles of Internal Medicine. According to the reference, cancer is defined the following two properties: uncontrolled cell growth and the capacity to invade or metastasize distant sites. The reference also recognizes that cancer is a genetic disease and genetic lesions or mutations lead to the malignant phenotype. This definition of cancer is accepted by those of skill and distinguishes the disease from *e.g.*, proliferative diseases that are not malignant and genetic diseases that do not affect cell proliferation. Therefore, one of skill would regard cancer as a specific disease. Thus, the asserted utility is sufficient to meet the requirement of 35 U.S.C. §101.

The Office Action also asserts that the specification lacks teaching of how Mkinase could be used to determine diagnostic or prognostic information about cancer. This is also incorrect. Disclosure of the use of Mkinase nucleic acids to determine information on diagnosis or prognosis of cancer is found in the specification at pages 40, line 12 through page 41, line 30. The specification specifically defines cancer as a "cell cycle associated disorder" or "disease state" at page 40, lines 13-14. Mkinase is defined as a cell cycle protein at page 4, lines 28-32.

For example, at page 41, line 8 the specification discloses that a cell cycle gene (Mkinase) of a patient can be sequenced and compared to the sequence of a known cell cycle

gene (wild type Mkinase). The specification goes on to disclose that differences in sequence between the patient's gene and the known cell cycle gene is indicative of a disease state or of a propensity of a disease state. The specification further discloses that differences in Mkinase expression level between a diseased tissue of a patient and an undiseased tissue from the patient or a different individual can be used to determine diagnostic or prognostic information about the disease condition (cancer). *See, e.g.* specification at page 41, lines 13-22. Thus, the specification does teach how Mkinase nucleic acids can be used in a diagnostic or prognostic way.

The Office Action also alleges that the Traf4 Mkinase association does not provide any information to the public as to how to actually use it to affect or diagnose cancer. Applicants respectfully traverse and direct the Examiner's attention to the declaration of Dr. Hitoshi. Dr. Hitoshi asserts that based on the discovery of Mkinase associated kinase activity and the binding of the Mkinase to TRAF4, those of skill would believe that the Mkinase protein was involved in tumorigenesis. Therefore, the specification does provide the required information to support the asserted utility of the claimed Mkinase nucleic acids.

Finally, as asserted in Dr. Hitoshi's declaration, the Mkinase neucleic acids were identified in a two hybrid screen using TRAF4 as bait. The two hybrid screen is accepted by those of skill in the art as a method to identify protein-protein binding. *See, e.g.*, Exhibit F Miller and Stagljar, *Methods in Mole. Biol.* 261:247-262 (2004). Miller and Stagljar disclose that the two hybrid method is a powerful method and one of the best methods to identify interacting proteins. "Furthermore, novel interacting partners can be found by screening a single protein or domain against a library of other proteins using this system. It is this latter feature-the ability to search for interacting proteins without any prior knowledge of the identity of such proteins- that is the most powerful application of the two-hybrid technique." Miller and Stagljar, p. 247 abstract. ". . . [W]hen an investigator has a favorite protein and wants to find out what other proteins it interacts with, the two-hybrid system is one of the best ways to start searching for those proteins. *Id.* at page 253. The Miller and Stagljar reference demonstrate that those of skill believe that two-hybrid results are indicative of functional interaction between proteins.

The specification discloses that TRAF4 is expressed in epithelial stem cells and not expressed in differentiated or transformed epithelial cells and that TRAF4 is expressed in breast carcinoma cells. See, *e.g.*, specification at page 5, lines 1-5. Binding of TRAF4 by Mkinase is also disclosed in the specification. See *e.g.*, specification at page 27, lines 20-22. Labeling of Mkinase protein is disclosed in the specification at page 20, lines 1-6. Thus, labeled Mkinase can be used to bind to and detect TRAF4 protein in cells. In addition, in some cell type, *e.g.*, breast carcinoma cells, labeled Mkinase can be used to determine diagnosis or prognosis of cancer by detecting TRAF4 protein.

In view of the above arguments and remarks, withdrawal of the rejection under 35 U.S.C. §101 is respectfully requested.

II. Rejections under 35 U.S.C. §112, first paragraph, enablement

Claims 25 and 27-33 are rejected under 35 U.S.C. §112, first paragraph as allegedly lacking enablement. Specifically, the Office Action alleges that the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility, and that because of the alleged lack of utility, one of skill would not know how the use the claimed invention.

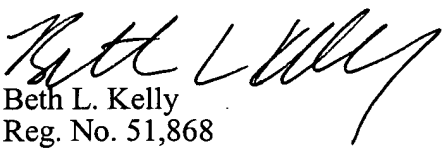
Applicants have submitted arguments in support of the Mkinase utility asserted in the application as filed in Section I of this response. In view of those arguments, Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph also be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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Attachments

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 4915
Ying Luo et al.	Examiner: Janet L. Andres
Application No.: 10/088,961	Technology Center/Art Unit: 1646
Filed: December 30, 2002	DECLARATION UNDER 37 C.F.R § 1.132 OF DR. YASUMICHI HITOSHI
For: TRAF4 ASSOCIATED CELL CYCLE PROTEINS, COMPOSITIONS AND METHODS OF USE	
Customer No.: 20350	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Yasumichi Hitoshi, M.D., Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my medical degree from Kumamoto University Medical School in 1987. I received a Ph.D. in immunology from The Institute for Medical Immunology, Kumamoto University Medical School in 1991. I was a postgraduate research associate at the Institute for Medical Immunology, Kumamoto University Medical School in 1991 and at the Institute of Medical Science, The University of Tokyo from 1992-1995. From 1995-1998 I was a postdoctoral fellow in the Department of Molecular Pharmacology at Stanford University. A copy of my curriculum vitae is attached hereto as Exhibit D.

EXHIBIT A

3. I have worked in the department of Cell Biology at Rigel Pharmaceuticals, Inc. since 1998. Currently, I am Director and Project Leader at Rigel Pharmaceuticals, Inc.

4. The present invention claims Mkinase protein, a cell cycle protein that binds to the TRAF4 protein.

5. I have read and am familiar with the contents of the patent application. In addition, I have read the Office Action, mailed June 9, 2004, received in the present case. It is my understanding that the Examiner believes that the present invention does not provide a "specific and substantial" use for the claimed nucleic acids. This declaration is provided to demonstrate that the Mkinase cell cycle protein has "specific and substantial" utility based on the specification and the utility of a closely related NTKL protein.

6. The Mkinase protein was identified by Scientists at Rigel Pharmaceuticals in a two hybrid screen using the TRAF4 protein as "bait." Thus, Mkinase binds to the TRAF4 protein. The TRAF4 protein is overexpressed in some cancer cells and is recognized by those of skill to play a role in tumorigenesis by regulating signal transduction pathways. It is my opinion that those of skill would believe that the Mkinase protein has a role in tumorigenesis and signal transduction based on its ability to bind to the TRAF4 protein.

7. The Mkinase protein is closely related to the NTKL protein. I have read a reference by Kato *et al.* that describes identification and characterization of the NTKL protein. (Kato *et al*, *Genomics* 79:760-767 (2002), enclosed as Exhibit B.) According to Kato *et al.*, the NTKL protein contains a conserved kinase domain and maps to a breakpoint region on chromosome 11 that is associated with cancer. I have also compared the sequence of the Mkinase protein to the sequence of the NTKL protein and the sequence alignment is submitted as Exhibit C. The alignment indicates that the NTKL sequence and the Mkinase sequence share 99% identity. Based on Kato *et al.* and comparison of the NTKL and Mkinase amino acid sequences, I believe it more likely than not that the Mkinase gene is useful as a diagnostic for cancer.

8. At the time of filing, the kinase domain of the Mkinase protein was identified by the Applicants using well-known sequence comparison programs. *See, e.g.*, specification at page 4, lines 13-15; page 6, line 26 through page 7, line 27; page 56, lines 16-20; and Figure 7A and 7B. Also at the time of filing, Applicants disclosed that the Mkinase protein binds to the TRAF4 protein, which was recognized to have a role in tumorigenesis. In my opinion, the Kato *et al.* reference described above confirms the identification of the Mkinase protein as a protein or gene product associated with cancer, as asserted in the specification. Also in my opinion, the discovery of the Mkinase associated kinase activity and the binding of Mkinase to the TRAF4 protein would lead those of skill to believe that the Mkinase protein was a protein involved in tumorigenesis.

9. In view of the foregoing, it is my scientific opinion that one of skill in the art, at the time the application was filed, would recognize the utility of the Mkinase proteins of the present invention.

Date: 12/7/04 By: _____

Yasumichi Hitoshi, M.D., Ph.D.

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Identification and Characterization of the Human Protein Kinase-like Gene *NTKL*: Mitosis-Specific Centrosomal Localization of an Alternatively Spliced Isoform

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Although the centrosome has an essential role in mitosis, its molecular components have not been fully elucidated. Here, we describe the molecular cloning and characterization of the human gene *NTKL*, which encodes an evolutionarily conserved kinase-like protein. *NTKL* mRNA is found ubiquitously in human tissues. *NTKL* is located on 11q13 and is mapped around chromosomal breakpoints found in several carcinomas, suggesting that *NTKL* dysfunction may be involved in carcinogenesis. Alternative splicing generates two variant forms of *NTKL* mRNA that encode protein isoforms with internal deletions. When fused to green fluorescent protein, the full-length product and one of the variant proteins are found in cytoplasm. The other variant product also exists in the cytoplasm during interphase, but is found in the centrosomes during mitosis. Endogenous NTKL protein is also localized to the centrosomes during mitosis. This cell-cycle-dependent centrosomal localization suggests that *NTKL* is involved in centrosome-related cellular functions.

Key Words: *NTKL*, *Homo sapiens*, protein kinase, alternative splicing, gene family, centrosome, mitosis, multimer formation

INTRODUCTION

Mitotic cell division requires tight control of spindle formation and the subsequent segregation of condensed chromosomes to ensure faithful transmission of entire genomes to daughter cells. The centrosome, composed of a pair of centrioles and associated electron-dense pericentriolar materials, has an essential role in this process. The centrosome lies close to the nucleus at interphase and undergoes duplication that is linked to the onset of DNA replication at the G1/S transition. At the end of the G2 phase, the duplicated centrosomes migrate to opposite sides of the nucleus. At mitosis, cytoplasmic microtubules are organized into bipolar spindles. The centrosome functions as a microtubule-organizing center and has a critical role in accurate chromosome segregation [1,2].

Defects in centrosome function lead to aberrant chromosomal segregation and changes in chromosome numbers and structure [3]. These chromosomal abnormalities are well recognized as the predominant class of genetic instability found in cancer cells [4] and are also believed to promote the abnormal growth and metastasis of cancer cells [5]. Indeed, various human carcinomas exhibit hypertrophy of the centrosomes [6–8]. Therefore, it is believed that the determination of centrosome function at the molecular level will contribute to the understanding of how carcinomas develop.

The importance of the centrosome has been recognized, but its molecular components have not been fully characterized. The γ-tubulin protein is a major component of the centriole core and is essential for microtubule nucleation [9]. The centrosome is also associated with regulatory factors, such as TP53 [10], RB1 [11], CCNB1 [12], CDC2 [13], and BRCA1 [14], in cell-cycle-dependent manners. Increasing numbers of protein kinases have been reported to be located at the centrosome [15]. These include aurora-related kinases required for centrosome separation and mitotic spindle assembly [16], polo-like kinases involved in centrosome maturation and bipolar spindle formation [17], and NIMA-related kinases that regulate centrosome separation [15]. Some of these genes are overexpressed in human carcinomas [18–21], suggesting the possible involvement of abnormal regulation of centrosomal kinases in carcinogenesis and tumor progression.

Here, we describe the molecular cloning and characterization of *NTKL*, which encodes a protein with similarity to protein kinases at its amino-terminal region. Searching DNA databases revealed that *NTKL* is a member of a large family found in a broad range of eukaryotes. We identified three

EXHIBIT B

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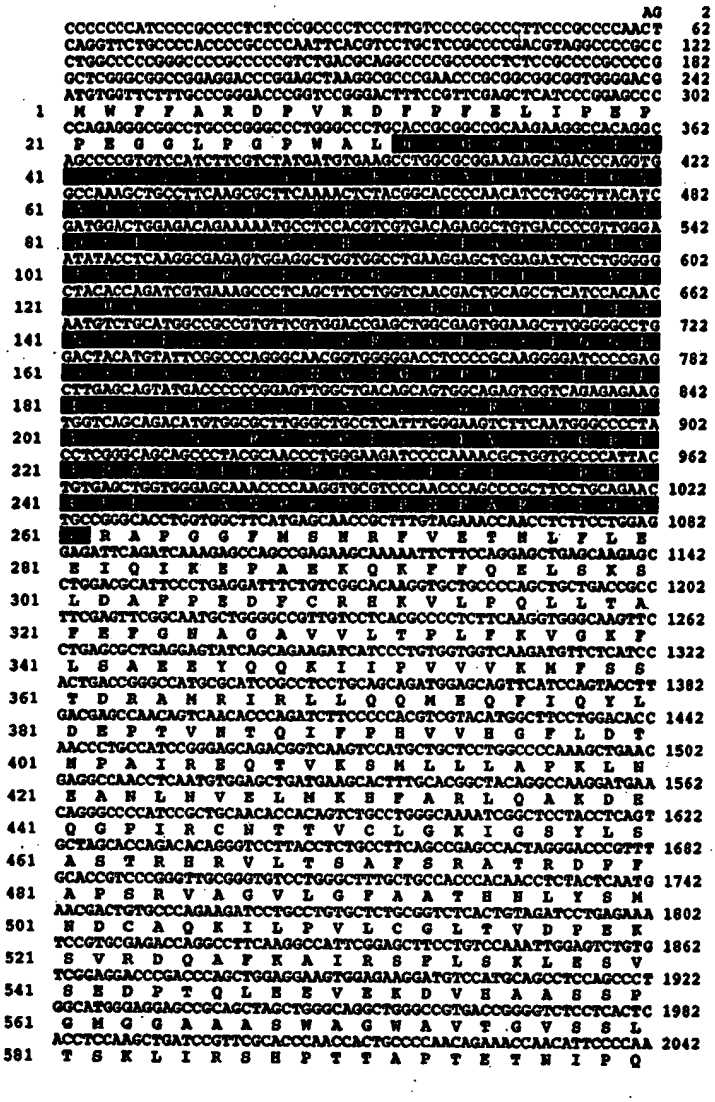

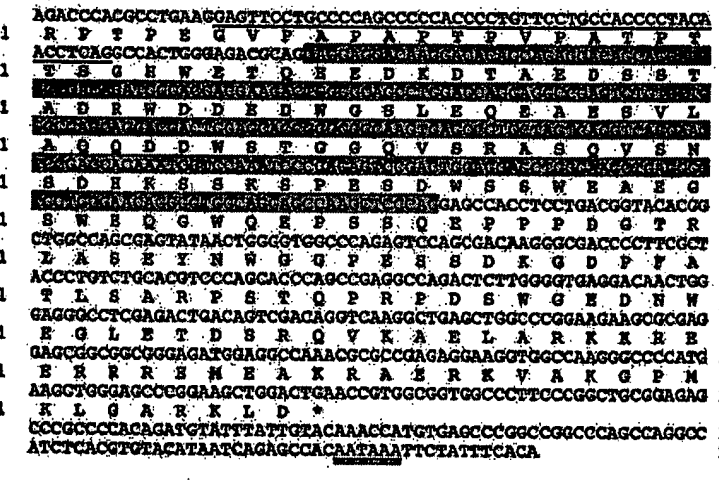

FIG. 1. Nucleotide and deduced amino acid sequences of human *NTKL* cDNA. Nucleotides and amino acids are numbered at the right and left sides, respectively. The protein kinase-like domain is shaded in black. The underlined area indicates the region deleted in both variant 1 and variant 2. The region spliced out in variant 2 is shown in gray. A possible poly(A) signal is double-underlined.

NTKL cDNA isoforms generated by alternative splicing. One of the isoform proteins was localized to the centrosomes during mitosis. Our observations suggest the probable involvement of *NTKL* in centrosome functions.

RESULTS

Isolation, Sequence Analysis, and Expression Profile of *NTKL*

In the course of large-scale sequencing of a human mammary gland cDNA library, we isolated cDNA clones that seemed to encode a novel kinase-like protein. We also isolated two additional cDNA clones with internal deletions in the 3′ regions. We designated these clones as full-length, variant 1, and variant 2, respectively. (We first named this novel gene *GKLP* and deposited the nucleotide sequences in DDBJ/EMBL/GenBank, but later changed the gene name from *GKLP* to *NTKL* (N-terminal kinase-like) on a recommendation by the HUGO Gene Nomenclature Committee.)

To obtain the entire open reading frame, we designed nested primers from the nucleotide sequence of the *NTKL* cDNA clone and carried out 5′-RACE using mammary gland cDNA. Two successive rounds of PCR using the nested primers yielded an 800-bp cDNA fragment. Sequence analyses of the cDNA clones and the cDNA fragment isolated by 5′-RACE resulted in the assembly of a single, large, open reading frame (Fig. 1). This open reading frame started from a strong consensus initiation sequence and encoded a putative protein of 808 amino acids with a predicted molecular mass of 89.6 kDa. The putative protein encoded by the variant 1 cDNA consisted of 791 amino acids and lacked the region corresponding to amino acids 606–622 of the full-length *NTKL*. Variant 2 consisted of 707 amino acids and lacked two corresponding regions of the full-length *NTKL* (amino acids 606–622 and 629–712). Analysis of the deduced primary sequence using the Conserved Domain program suggested that the NTKL protein contains a

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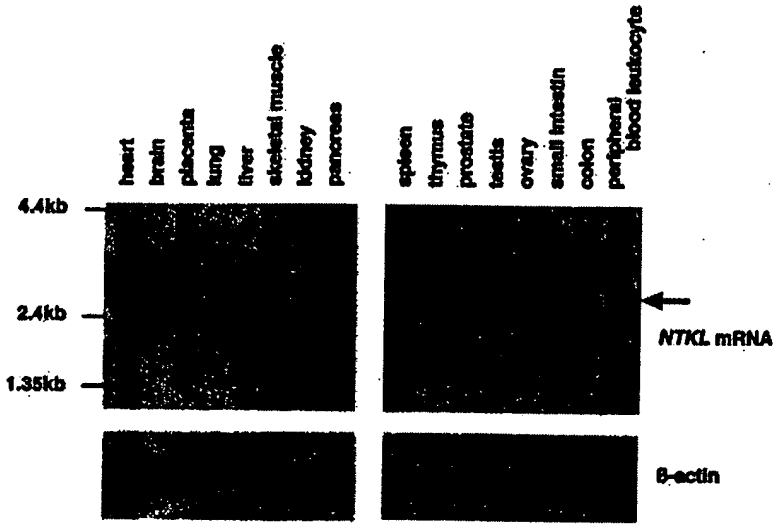

FIG. 2. Tissue distribution of *NTKL* mRNA. NTKL cDNA was labeled with [α-^{32}P]dCTP by random priming and hybridized to a multiple tissue northern blot membrane. In the lower panel, expression of β-actin mRNA is shown as a loading control.

kinase-like domain in the N-terminal region (amino acids 32–261). A cluster of basic amino acid residues was found in the carboxy-terminal region. No other protein motifs were observed in the deduced primary structure.

We then examined the tissue distribution of *NTKL* mRNA. We carried out northern blot analysis using a multiple-tissue blot membrane and detected 2.8-kb *NTKL* mRNA in all tissues (Fig. 2).

Evolutionary Conservation of *NTKL*

Searching the GenBank database with NTKL sequence revealed several proteins with significant homology to human NTKL (Fig. 3). Human NTKL is most closely related to the mouse NTKL homolog (90% identity at the amino acid level), which was originally isolated as a factor interacting with protein kinase B [22]. Proteins structurally similar to human NTKL were found in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*. These proteins carry protein kinase-like domains in the N-terminal regions. YOR112W of *Saccharomyces cerevisiae* is predicted to encode an NTKL-related protein that shows no significant similarity to protein kinases.

Genomic Structure and Alternative Splicing of *NTKL*

Searching by BLAST, we found that the nucleotide sequence of the *NTKL* cDNA matched two human genome draft sequences (GenBank acc. nos. AF255613 and Hs11_25998). To investigate the genomic organization of *NTKL*, we compared the cDNA and genomic sequences. *NTKL* was mapped to chromosome 11q13 and spanned approximately 15 kb between microsatellite markers *D11S4933* and *D11S546*. This genomic region is known to contain breakpoints for chromosomal translocations reported in two cases of extragonadal germ cell tumors and in one case of renal cell carcinoma [23–25]. A detailed sequence analysis of the genomic region between *D11S4933* and *D11S546* on 11p13 has been reported [26]. *NTKL* was located in the genomic region where the breakpoints are suspected to exist [26] (data not shown), although the precise position of the chromosomal breakpoints is yet to be determined.

NTKL is composed of 18 exons (Fig. 4A). As described above, we isolated two C-terminal variants that seemed to be generated by alternative splicing. Variant 1 lacks the 5′-half of exon 14. Variant 2 uses different splice acceptor and donor sites in exon 14, skips over the entire exon 15, and lacks the 5′ half of exon 16 (Fig. 4B). As the translational reading frames of these splicing variants are the same as the full-length *NTKL* mRNA, the two variant mRNAs encode proteins with internal deletions (Fig. 1).

Biochemical Characterization of NTKL

To investigate the function of NTKL, we searched for proteins that interact with NTKL by yeast two-hybrid screening of a human mammary gland cDNA library. We isolated several positive clones that were then found to be also *NTKL* (data not shown). To examine the multimer formation of NTKL in mammalian cells, we coexpressed FLAG- and hemagglutinin epitope (HA)-tagged NTKL proteins in COS7 cells and performed immunoprecipitation with an anti-FLAG antibody. HA-tagged NTKL coprecipitated with FLAG-NTKL, confirming the multimer formation of NTKL *in vivo* (Fig. 5A).

To examine the state of the endogenous NTKL protein, we raised an antibody against NTKL. The antibody showed highly specific recognition of the endogenous NTKL protein in western blot analysis (Fig. 5B). The mobility of the endogenous NTKL protein was slower than the calculated molecular weight. The FLAG-tagged NTKL protein, which was expressed from the plasmid, also showed slower mobility (data not shown), suggesting that NTKL protein is modified posttranslationally. We then examined the multimer formation of the endogenous NTKL protein. Whole-cell extract was prepared from MCF7 and reacted with bis(sulfosuccinimidyl)suberate (BS3), a bipolar cross-linking reagent. The monomeric form of the NTKL protein was converted to the larger species (approximately 300 kDa) in a BS3 concentration-dependent manner (Fig. 5C). Similar results were obtained with another cross-linking reagent, disuccinimidyl suberate (data not shown). These observations were consistent with the complex formation of the exogenously expressed NTKL proteins in COS7 and the NTKL–NTKL interaction in yeast two-hybrid assays. We therefore concluded that the NTKL protein, at least in part, forms a multimer, most likely a trimer.

Next, we examined whether NTKL possessed protein kinase activity based on its sequence similarity to protein kinases. However, immunoprecipitated FLAG-tagged NTKL

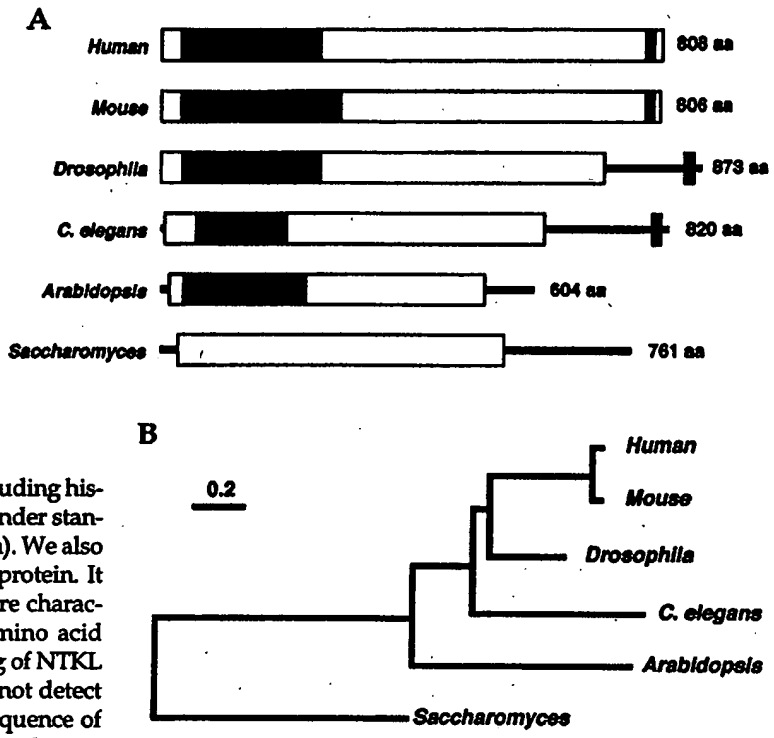

FIG. 3. Evolutionary conservation of *NTKL*. (A) Schematic representation of NTKL-related proteins. BLAST search using the human *NTKL* cDNA sequence against the GenBank database revealed the existence of open reading frames that are predicted to encode NTKL-related proteins in the following eukaryotic species: YOR112 of *S. cerevisiae* (GenBank acc. no. CAA99310), At2g40730 of *A. thaliana* (AC007660), W07G4.3 of *C. elegans* (CAB01444), and CG1973 of *Drosophila* (AAF56933). Murine *NTKL* homolog (AF276514) was also detected. Structures of human NTKL and related proteins are shown. Boxes indicate regions of similarity to the human NTKL. Regions with similarity to protein kinases are shown in black. Gray boxes show clusters of basic amino acids. Thick bars indicate regions that have no similarity to human NTKL protein. (B) Phylogenic tree of NTKL-related proteins. The phylogenic relationships of the NTKL-related proteins were analyzed using ClustalW. The phylogenic tree was depicted using the DendroMaker program.

protein did not phosphorylate purified proteins, including histone H1, enolase, myelin basic protein, and casein, under standard *in vitro* kinase assay conditions (data not shown). We also failed to detect autophosphorylation of the NTKL protein. It has been proposed that almost all protein kinases are characterized by 12 subdomains, in which important amino acid residues are well conserved [27]. The mouse homolog of NTKL lacks one subdomain (subdomain I) [22]. We could not detect this subdomain motif in the deduced amino acid sequence of human NTKL. Together with the results of *in vitro* kinase assays, we concluded that while human NTKL exhibits a protein kinase-like structure, it does not possess kinase activity.

Centrosomal Localization of the NTKL Variant 2-EGFP and Endogenous NTKL during Mitosis

To identify a possible site of action for the NTKL protein and to obtain information about its function, we examined the subcellular localization of NTKL during the cell cycle. We constructed plasmids that expressed each NTKL isoform as a fusion to enhanced green fluorescent protein (EGFP). The plasmids were introduced into MCF7 cells. The subcellular localization of the fusion proteins was observed by fluorescence microscopy. Full-length and variant 1 NTKL-EGFP fusion proteins were localized to the cytoplasm throughout the cell cycle (data not shown). However, the variant 2-EGFP fusion protein exhibited mitosis-specific localization to centrosomes (Fig. 6). During interphase, variant 2-EGFP was also found in cytoplasm, similar to full-length and variant 1 products (data not shown). At the beginning of mitosis, a pair of bright green foci was observed in the nucleus that emerged as the chromosomes became condensed (Figs. 6A–6D). At prometaphase, the foci migrated to opposite poles of the nucleus (Figs. 6E and 6F). We also stained the transfected cells with an anti-α-tubulin antibody and found that the foci of the variant 2-EGFP fluorescence were localized to the spindle poles. During chromosomal segregation, variant 2-EGFP comigrated with the spindle poles (Figs. 6G–6P). In addition to the punctate staining at the

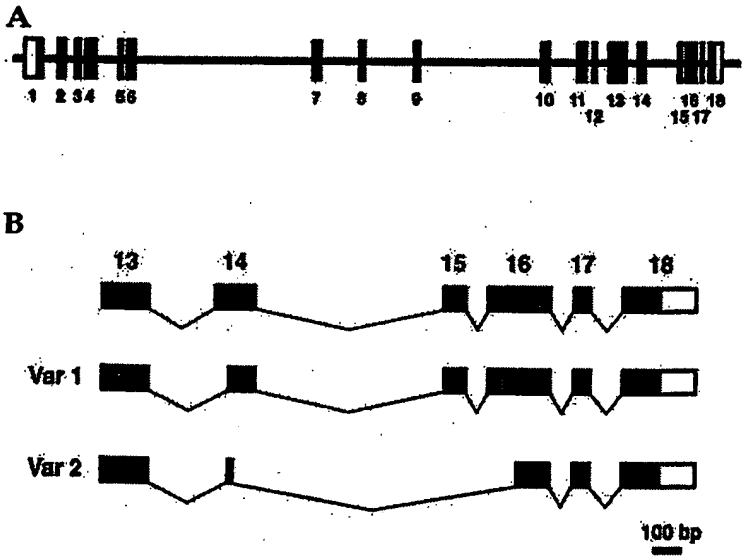

FIG. 4. Genomic organization of *NTKL*. (A) Structure of the human *NTKL* gene. Exons are numbered and shown as boxes. Protein-coding regions are shaded. The 5′- and 3′-untranslated regions are shown by open boxes. (B) Alternative splicing of *NTKL*. Exons 13–18 are highlighted. Full-length *NTKL* mRNA comprises 18 exons. Splicing variant 1 lacks the 5′ half of exon 14. Splicing variant 2 uses different splice donor and acceptor sites in exon 14, and skips over exon 15 and the 5′-half of exon 16. Nucleotide sequences skipped in the splicing variants are shown in Fig. 1.

doi:10.1006/geno.2002.6774, available online at http://www.idealibrary.com on IDEAL

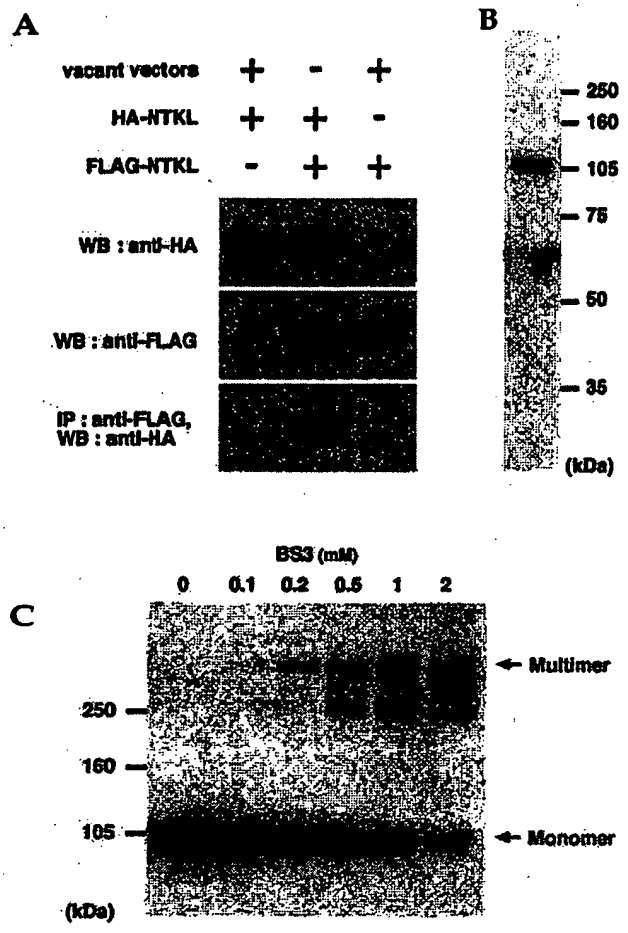

FIG. 5. Multimer formation of the NTKL protein. (A) Complex formation exogenously expressed FLAG- and HA-tagged NTKL proteins. COS7 cells were cotransfected with combinations of pFLAG-NTKL, pHA-NTKL, and vacant vectors as indicated at the top of the panels. Immunoprecipitation was carried out with anti-FLAG monoclonal antibody. Complex formation of the FLAG-tagged and HA-tagged NTKL proteins was analyzed by western blot. (B) Detection of endogenous NTKL protein by western blot. Whole-cell extract of MCF7 was subjected to SDS-PAGE followed by western blot with the anti-NTKL antibody. (C) Cross-link analysis of the endogenous NTKL protein. A bipolar cross-linking reagent BS3 was reacted with the whole-cell extract of MCF7 at the indicated concentrations. Multimer formation was analyzed by SDS-PAGE followed by western blot with the anti-NTKL antibody.

centrosomes, diffuse cytoplasmic fluorescence was also observed throughout mitosis. The cells transfected with EGFP alone showed no fluorescent foci at the centrosomes (data not shown). In the presence of nocodazole, the centrosomal localization was observed (Figs. 6Q–6R), suggesting microtubule polymerization is not required for the centrosomal localization of the variant 2–EGFP. Similar microtubule-independence was reported in several centrosomal proteins [28–30].

Next, we examined the subcellular localization of endogenous NTKL. The MCF7 cells were fixed in methanol/acetone mixture and stained with the anti-NTKL antibody. In this fixation condition, the signals for cytoplasmic NTKL protein were reduced, probably because soluble cytoplasmic NTKL protein was washed out. The endogenous NTKL protein was concentrated around the centrosomes at the mitotic stage (Figs. 6U and 6V). Finally, we performed the immunostaining with another cell line, HeLa, and observed similar centrosomal staining of the endogenous NTKL protein (Figs. 6W and 6X). These observations indicated that a fraction of endogenous NTKL is concentrated to the centrosomes during mitosis.

DISCUSSION

During the large-scale sequencing of human mammary gland cDNA, we isolated *NTKL* cDNA clones and two *NTKL* cDNA variants generated by alternative splicing. *NTKL* encoded a protein with sequence similarity to protein kinases in the N-terminal region. Searching the GenBank database, we found that several genes from a broad range of eukaryotes encoded proteins structurally related to human NTKL. To obtain insights into *NTKL* function, we examined the subcellular localization of the NTKL isoforms. While full-length and variant 1 NTKL proteins fused to EGFP were found in the cytoplasm throughout the cell cycle, NTKL variant 2–EGFP was concentrated to the centrosomes during mitosis in addition to being present in the cytoplasm during interphase. By using the anti-NTKL antibody, we observed that a fraction of endogenous NTKL was concentrated to the centrosomes during mitosis.

Although the primary structure of the NTKL protein showed significant similarity to protein kinases, immunoprecipitated FLAG-tagged NTKL protein did not exhibit kinase activity, despite the use of several purified proteins as substrates for the *in vitro* kinase assays. A subdomain shared by almost all protein kinases was absent from the human NTKL protein. As some NTKL-related proteins from other species also lack this subdomain motif, it is possible that human *NTKL* and the other related genes may derive from a common ancestral gene that once encoded a protein kinase but then evolved to lose kinase activity. A prime example of such differential evolution from an ancestral gene is *GAL1* and *GAL3* of *Saccharomyces cerevisiae*. The high similarity at the amino acid level suggests that *GAL1* and *GAL3* evolved from a single ancestral gene. However, Gal1 protein functions as a galactokinase that phosphorylates galactose, whereas Gal3 protein has no kinase activity but functions as a galactose sensor [31,32]. It is thought that the Gal3 protein first gained a novel function as a galactose sensor and then lost its kinase activity during evolution. Thus, it is possible that *NTKL* family members may have gained some new function and then lost their protein kinase activities. It is also possible that NTKL might have some protein kinase-related function, such as recognition and binding of phosphorylated proteins.

Another example for kinase-independent functions was reported in kinase suppressor of Ras (KSR). KSR shares

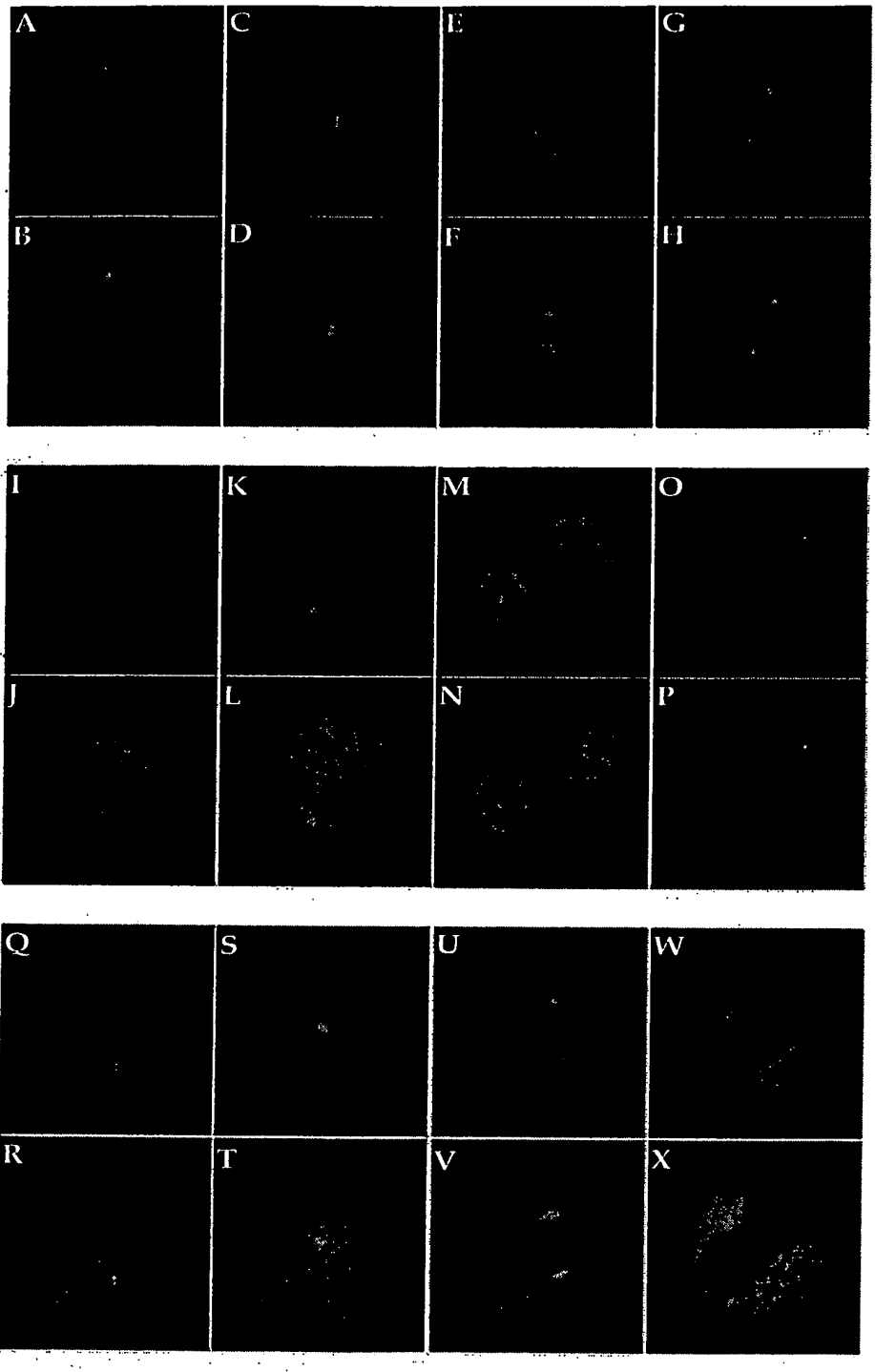

FIG. 6. Subcellular localization of NTKL variant 2-EGFP and endogenous NTKL. (A–P) Subcellular localization of NTKL variant 2-EGFP in MCF7 at mitosis. Cells were transfected with the NTKL variant 2-EGFP expression plasmid and treated with nocodazole at 150 ng/ml for 10 hours to increase the fraction of mitotic cells. Following washes with phosphate-buffered saline, mitosis was allowed to proceed for 75 minutes. Cells were then stained with anti-γ-tubulin antibody (red) and DAPI (blue). Subcellular localization of NTKL variant 2-EGFP was observed by fluorescence microscopy (A, C, E, G, I, K, M, and O). Images of α-tubulin staining were merged with those of NTKL variant 2-EGFP (B, D, F, H, J, L, N, and P). Stages of cell division are as follows: (A–D), prophase; (E) and (F), prometaphase; (G) and (H), metaphase; (I–L), anaphase; (M–P), telophase. (Q–T) Microtubule-independent centrosomal localization of the NTKL variant 2-EGFP. Subcellular localization of the NTKL variant 2-EGFP was observed in the presence (Q and R) or absence (S and T) of nocodazole (150 ng/ml) as above. (U–X) Centrosomal localization of the endogenous NTKL protein during mitosis. MCF7 (U and V) and HeLa (W and X) were double-stained with anti-NTKL (green) and anti-α-tubulin (red) antibodies. Nuclei were shown by staining with DAPI (blue).

sequence homology with Raf family kinases and has an important role in Ras-mediated signal transduction. KSR interacts with several components of the MAP kinase cascade to form a large protein complex. Similar to NTKL, KSR lacks several key properties of known protein kinases and functions in a kinase-activity-independent manner [33,34].

In this study, we identified three NTKL isoforms apparently derived by alternative splicing. Alternative splicing is an important means of generating protein diversity in eukaryotes. An increasing number of examples have been reported that alternative splicing can yield isoforms with different subcellular localization. For example, the nuclear mitotic apparatus protein NuMA1 is composed of three isoforms derived by alternative splicing, and these isoforms are differentially localized to the nucleus and centrosome [35]. Differential localization of alternative splicing-derived isoforms in the nucleus and cytoplasm has also been reported for a number of other proteins, including FGF3 [36], DNTT [37], NF2 [38], and BACH1 [39]. In *BCL2L1*, alternative splicing generates long and short transcripts that encode proteins with positive and negative effects on apoptosis, respectively [40]. In the case of NTKL, the full-length and variant 1 products are present in the cytoplasm throughout the cell cycle, whereas variant 2 product is concentrated to centrosomes during mitosis. The differential subcellular localization of the NTKL

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isoforms might reflect functional differences among the isoforms. It is also an interesting issue whether the alternative splicing of *NTKL* is regulated in cell-cycle-, developmental-stage-, or tissue-dependent manners.

The evolutionary conservation of *NTKL* among a wide range of eukaryotic species raises the possibility that *NTKL* might have a fundamental role in a cellular function common to eukaryotic cells. This speculation is supported by the observation that the expression of *NTKL* mRNA was virtually ubiquitous in all human tissues tested. In this respect, it is noteworthy that the variant 2 protein was concentrated to centrosomes at mitosis, which suggests that the NTKL variant 2 protein may have a mitosis-related function, such as in spindle formation or segregation of condensed chromosomes. Recently, Liu *et al.* showed by cell fractionation that mouse NTKL protein was concentrated in the low-density microsomal fraction [22]. While this fraction contained Golgi apparatus, cytoskeletons, and other small cellular compartments, it was still unclear where mouse NTKL protein was localized during mitosis. While the differential subcellular localization of the NTKL isoforms might reflect specific cellular roles, the precise function of NTKL remains to be elucidated. The significance of NTKL multimer formation and the possible involvement of dysfunction of *NTKL* in carcinogenesis are also yet to be investigated. Further research on human *NTKL*, together with the analysis of *NTKL*-related genes in model organisms, is required to address these questions.

Materials and Methods

Cloning of NTKL cDNA. *NTKL* cDNA clones were isolated during the large-scale sequencing of cDNA clones from a Matchmaker human mammary gland cDNA library (Clontech, Palo Alto, CA). To obtain the 5′-end of the *NTKL* cDNA, 5′-RACE was carried out using Marathon-Ready human mammary gland cDNA (Clontech) according to the manufacturer's protocols. Primers used for 5′-RACE were R1, 5′-GGGGTCATACTGCTCAAGCT-3′, and R2, 5′-TGCCACTGCTGTCAGCCAAC-3′. The 5′-RACE yielded a 0.8-kb cDNA fragment, which was subcloned into the pT7Blue plasmid vector (Novagen, Madison, WI). Nucleotide sequences of the isolated cDNAs were determined using a CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA).

Computer analysis of NTKL sequence. Homology search was performed by BLAST using default parameters (http://www.ncbi.nlm.nih.gov/BLAST/). Exon–intron boundaries were determined by comparison of *NTKL* cDNA nucleotide sequences to human draft sequences (GenBank acc. no. AF255613 and Hs11_25998). Protein motifs in the deduced amino acid sequence were predicted by searching the Conserved Domain Database with reverse position-specific BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Phylogenic relationships among human NTKL and related proteins were assessed using the ClustalW program at DDBJ (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html) and shown as a phylogenic tree by DendroMaker (http://www.cib.nig.ac.jp/dda/timanish/dendromaker/home.html).

Plasmid constructs. *NTKL* cDNA clones isolated from the human mammary gland cDNA library were ligated in-frame with the cDNA fragment obtained by 5′-RACE to generate entire cDNAs of the isoforms. The entire cDNA of each isoform was subcloned into the pEGFP-C (Clontech), pFLAG-CMV2 (Sigma, St. Louis, MO), and pcDNAHA plasmids, respectively. pcDNAHA was constructed by replacing the Xpress epitope of the pcDNA 3.1His (Invitrogen, Carlsbad, CA) with an HA epitope. All plasmids constructed in this study were verified by sequencing as described above.

Northern hybridization. *NTKL* cDNA was labeled with [α-^{32}P]dCTP by random priming using the Megaprime DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocols. The probe was hybridized to a human multiple tissue northern membrane (Clontech) according to the manufacturer's protocols. Signals for *NTKL* mRNA were detected by autoradiography. The membrane was reprobed with ^{32}P-labeled β-actin cDNA to show equivalent loading of poly(A)$^+$ RNA in each lane.

Antibody production. A cDNA fragment corresponding to the amino acids 484–707 of *NTKL* variant 2 was subcloned in-frame into pGEX plasmid (Amersham Pharmacia Biotech). A glutathione S-transferase (GST)-NTKL fusion protein was expressed in *Escherichia coli* BL21(DE3) and purified with glutathione-agarose beads (Amersham Pharmacia Biotech). Anti-NTKL antibody was raised by immunizing rabbits with the GST-NTKL protein (Takara Shuzo, Ohtsu, Shiga, Japan) and purified with protein A-agarose (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Immunoprecipitation and western blot. The COS7 green monkey kidney cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (A9909, Sigma). For immunoprecipitation analyses, COS7 cells at 70% confluency were cotransfected with FLAG- and HA-NTKL expression plasmids by the lipofection method using the TransIT LT-1 reagent (Panvera, Madison, WI) as described [41]. Twenty-four hours after transfection, the cells were collected and lysed in extraction buffer containing 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, and Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). After centrifugation at 20,000*g* for 5 minutes, the whole-cell extracts were subjected to immunoprecipitation using anti-FLAG monoclonal antibody M2 (Sigma), and the interaction between FLAG- and HA-tagged NTKL was analyzed by western blot as described [42].

Chemical cross-linking. MCF7 human breast cancer cell line was obtained from the Cell Resource Center for Biomedical Research, Tohoku University, and maintained as described above. Cells were lysed in phosphate-buffered saline supplemented with 0.2% Triton X-100, and the whole-cell extract was cleared by centrifugation at 20,000*g* for 5 minutes. The whole-cell extract was reacted with bis(sulfosuccinimidyl)suberate (Pierce, Rockford, IL) at increasing concentrations for 20 minutes on ice. Reactions were stopped by adding 50 mM Tris-Cl, pH 6.8, followed by incubation on ice for 15 minutes. Covalent cross-linking of the endogenous NTKL protein was analyzed by SDS-PAGE followed by western blot using anti-NTKL antibody as described above.

Immunofluorescence microscopy. MCF7 cells were seeded onto culture slides (Becton Dickinson, Bedford, MA) and transfected with the pEGFP-NTKL plasmid, as described [41]. At 1 hour after transfection, the culture medium was replaced with fresh medium. After a further incubation of 20 hours, nocodazole (Sigma) was added to the medium at 150 ng/ml to increase the fraction of mitotic cells. After 10 hours, the cells were washed twice with phosphate-buffered saline (PBS) and incubated in fresh medium for 75 minutes. The cells were fixed, permeabilized, and stained with anti-α-tubulin monoclonal antibody B512 at 15 ng/ml (Sigma) and anti-mouse antibody conjugated with Alexa 594 at 100 ng/ml (Molecular Probes, Eugene, OR) as described [41]. Stained cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 4, 6-diamino-2-phenylindole (DAPI). Fluorescence was observed using an Axioplan-2 microscope (Carl Zeiss, Jena, Germany) equipped with a Quips Smart Capture System (Vysis, Downers Grove, IL).

To examine subcellular localization of endogenous NTKL protein, MCF7 and HeLa were seeded on culture slides precoated with polylysine (Biocoat, Becton Dickinson). Cells were fixed for 10 minutes at −20°C in 33% methanol/67% acetone prechilled at −20°C. The fixed cells were double-stained with the anti-NTKL and anti-α-tubulin antibodies, and subjected to fluorescence microscopy as above.

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Sequence data from this article have been deposited in the DDBJ/EMBL/GenBank Data Libraries under accession numbers AB51427 (full-length NTKL), AB051428 (variant 1), and AB047077 (variant 2).

Align two sequences

Thu Dec 9 01:38:21 "GMT 2004

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>SEQ ID NO:1, 808 bases, AFE764FE checksum.          808 aa vs.
>NTKL AB051427, 808 bases, 1526818B checksum.       808 aa
scoring matrix: , gap penalties: -12/-2
99.5% identity;          Global alignment score: 5480

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               10        20        30        40        50        60

               70        80        90       100       110       120
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              130       140       150       160       170       180
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NTKL   LEQYDPPELADSSGRVVREKWSADMWRLGCLIWEVFNGPLPRAAALRNPGKIPKTLVPHY
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              250       260       270       280       290       300
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              310       320       330       340       350       360
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              430       440       450       460       470       480
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EXHIBIT C

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                  490       500       510       520       530       540
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                  550       560       570       580       590       600
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                  610       620       630       640       650       660
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                  670       680       690       700       710       720
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                  730       740       750       760       770       780
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                  730       740       750       760       770       780

                  790       800
/usr/t    ERRREMEAKRAERKVAKGPMKLGARKLD
          ::::::::::::::::::::::::::::
NTKL      ERRREMEAKRAERKVAKGPMKLGARKLD
                  790       800

Elapsed time:  0:00:00
```

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EXHIBIT D

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CELL BIOLOGY OF CANCER

Two characteristic features define a cancer: cell growth not regulated by external signals (i.e., autonomous) and the capacity to invade tissues and metastasize to and colonize distant sites (see Chap. 85). The first of these features, the uncontrolled growth of abnormal cells, is a property of all neoplasms, or new growths. A neoplasm may be benign or malignant. If invasion, the second cardinal feature of cancer, is present, the neoplasm is malignant. Cancer is a synonym for *malignant neoplasm.* Cancers of epithelial tissues are called *carcinomas*; cancers of nonepithelial (mesenchymal) tissues are called *sarcomas.*

Cancer is a genetic disease, but the level of its expression is the single cell. Although some forms of cancer are heritable, most mutations occur in somatic cells and are caused by intrinsic errors in DNA replication or are induced by carcinogen exposure. A single genetic lesion is usually not sufficient to induce neoplastic transformation of a cell. The malignant phenotype is acquired only after several (5 to 10) mutations (usually developing over many years) lead to derangements in a variety of gene products. Each genetic alteration may cause phenotypic changes typified by the progression in epithelial tissues from hyperplasia to adenoma to dysplasia to carcinoma in situ to invasive carcinoma. Resistance to neoplastic transformation is due to levels of control at every phase of cell function. Abnormalities in the function of one protein may be compensated by other proteins and pathways. An analogy can be drawn between the complex behavior of interacting signaling cascades and computer-based neural networks that can be adapted or trained to recognize patterns of complex inputs and respond to each pattern with a specific pattern of output.

The more than 200 discrete cell types in the body are not equally susceptible to developing cancer. Some cells, such as cardiac myocytes, sensory receptor cells for light and sound, and lens fibers, persist throughout life without dividing or being replaced. Neoplasia in such tissues is exceedingly rare. Most differentiated tissues undergo turnover characterized by cell death and replacement. When natural turnover rates are slow, fully differentiated cells may be induced to proliferate and produce fully differentiated daughter cells. For example, hepatocytes are capable of dividing to replace senescent, damaged, or surgically removed liver tissue.

In tissues with rapid turnover, such as skin, bone marrow, and gut, the differentiated function and the replacement function are carried out by different cell types. Under normal circumstances, an individual cell is on one of two largely mutually exclusive paths: division or differentiation. Cells capable of dividing are undifferentiated (stem cells), whereas terminally differentiated cells are unable to divide. Stem cells produce daughter cells that can either become new stem cells (thus replenishing the stem cell compartment) or undergo terminal differentiation, depending on the circumstances and the environmental signals. Stem cells are distinguished from differentiating cells by different patterns of gene expression. Gene expression is the product of the tissue-specific programming of gene expression interacting with environmental factors such as cell-to-cell contact; interactions with extracellular matrix; endocrine hormones; paracrine growth and differentiation factors; and stresses such as heat, oxidation, irradiation, and physical distortion or traction.

Cancer is most common in tissues with rapid turnover, especially those exposed to environmental carcinogens and whose proliferation is regulated by hormones. The most common genetic changes involve the activation of proto-oncogenes or the inactivation of tumor suppressor genes (see Chap. 84). Although genetic damage is nearly universal in human cancer, cells with neoplastic features can be generated in vitro without genetic damage. Removal and in vitro culture of cells from the epiblast of a murine embryo lead to the uncontrolled proliferation of the cells and the generation of a teratocarcinoma cell line capable of producing tumors when inoculated into animals. The removal of these normal embryonic cells from their normal environment leads to uncontrolled growth. However, if the teratocarcinoma cells are reinjected into an early embryo, under the inductive influence of their normal neighbors they can differentiate into normal organs and tissues appropriate for the location where they are injected.

Thus, environmental factors exert potent effects on the gene expression of target cells. The panoply of signals received by a particular cell leads to the activation of particular sets of transcription factors. The pattern of expression of transcription factors determines whether a cell will divide, differentiate, or die.

PRINCIPLES OF CELL CYCLE REGULATION

The mechanism of cell division is substantially the same in all dividing cells and has been conserved throughout evolution. The process assures that the cell accurately duplicates its contents, especially its chromosomes. The cell cycle is divided into four phases. During M phase, the replicated chromosomes are separated and packaged into two new nuclei by mitosis and the cytoplasm is divided between the two daughter cells by cytokinesis. The other three phases of the cell cycle are called *interphase*: G1 (gap 1), a period of growth during which the cell determines its readiness to commit to DNA synthesis; S (DNA synthesis), during which the genetic material is replicated and no rereplication is permitted; and G2 (gap 2), during which the fidelity of DNA replication is determined and errors are corrected.

During S phase, DNA synthesis begins with the unfolding of chromatin from the DNA and the addition of DNA helicase and single-strand binding proteins that help open the double helix. Replication origins are spaced roughly 100,000 nucleotide pairs apart throughout the genome. DNA polymerase and DNA primase attach to these sites and catalyze the polymerization of the DNA at a rate of about 50 nucleotides per second. Topoisomerases break and reseal DNA strands to prevent tangling. Although this system for replication is efficient and accurate, occasional mistakes are made, and these mistakes in the replicated sequences are repaired by a variety of mechanisms. In some cancers, the mismatch repair mechanisms are defective and errors are routinely passed along to daughter cells, increasing the development of new mutations. Once a DNA segment is replicated and the replication units reassembled, chromatin binds to the nascent DNA chain, assuring that each region is replicated only once. DNA polymerase is unable to replicate the end of a DNA chain completely. This problem has been solved by the addition of tandem repeats of a six-nucleotide sequence (GGGTTA) to the ends of each chromosome. These repeated

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Using the Yeast Two-Hybrid System to Identify Interacting Proteins

John Miller and Igor Stagljar

Abstract

The yeast two-hybrid system is a powerful technique for studying protein–protein interactions. Two proteins are separately fused to the independent DNA-binding and transcriptional activation domains of the Gal4p transcription factor. If the proteins interact, they reconstitute a functional Gal4p that activates expression of reporter gene(s). In this way, two individual proteins may be tested for their ability to interact, and a transcriptional readout can be measured to detect this interaction. Furthermore, novel interacting partners can be found by screening a single protein or domain against a library of other proteins using this system. It is this latter feature—the ability to search for interacting proteins without any prior knowledge of the identity of such proteins—that is the most powerful application of the two-hybrid technique.

Key Words

Two-hybrid methodology; protocol; library screening.

1. Introduction

Among the myriad methods for examining protein–protein interactions, the yeast two-hybrid system is one of the easiest and most inexpensive to perform. All that is required is a protein(s) of interest, the ability to clone into a vector, yeast, and various yeast media. The technique works by capitalizing on the modular nature of a transcription factor, Gal4p, which contains separable DNA-binding and transcriptional-activation domains *(1,2)*. The amino-terminal 147 residues ($Gal4_{1-147}$) of Gal4p code for the DNA-Binding Domain (BD) of the protein, while the last 114 residues ($Gal4_{768-881}$) encompass a strong transcription Activating Domain (AD). Fields and Song demonstrated that these separate domains of the Gal4p protein could stimulate transcription at a promoter that contained *cis* elements [upstream activating sequences (UAS)] recognized

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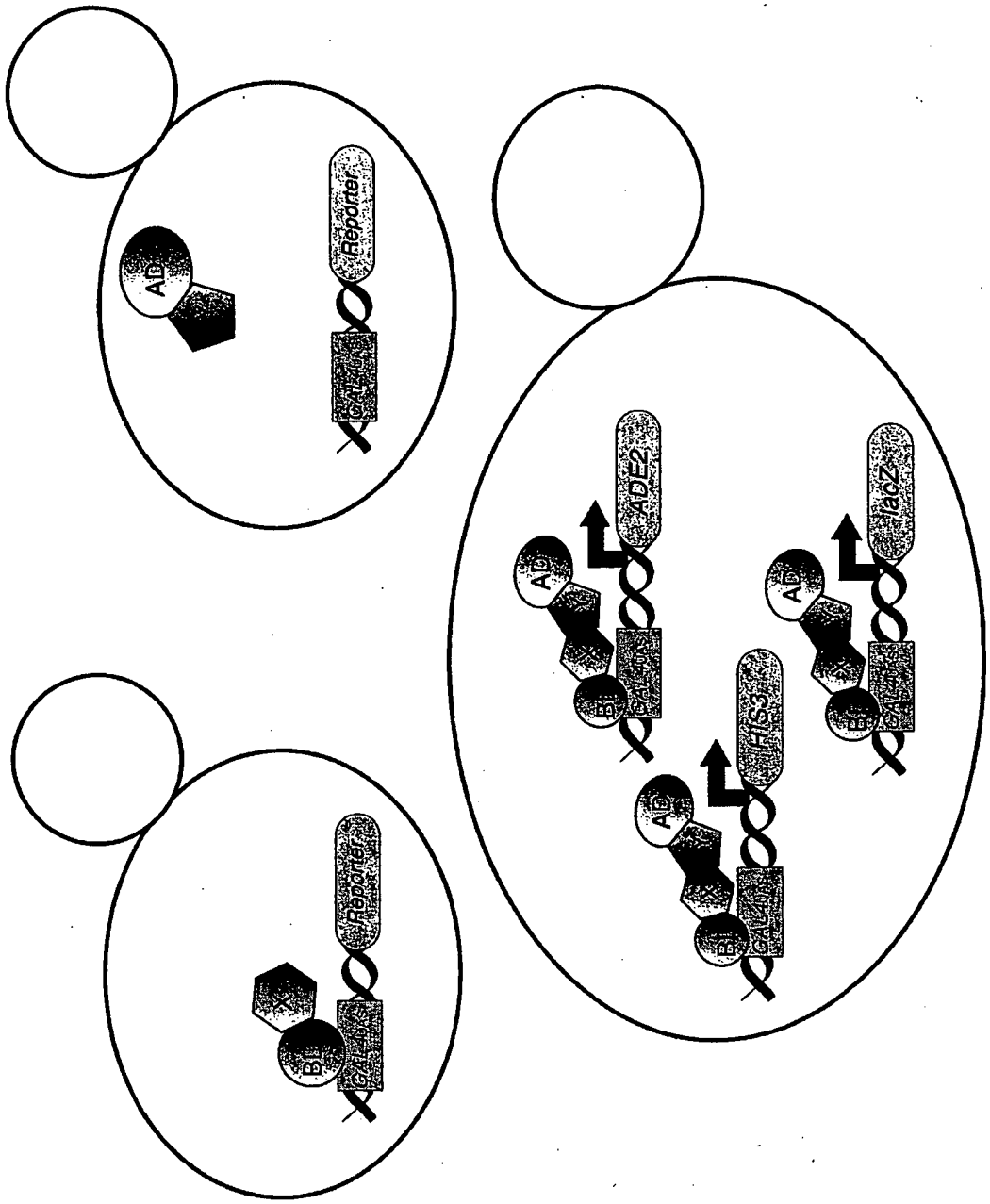

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by Gal4p if they were attached to two proteins that physically associate (**Fig. 1**) *(3)*. Therefore, the isolated BD and AD domains could potentially be fused to any two proteins, and if the proteins interact, then the transcription from genes bearing GAL4-UAS elements will report the interaction. BDs (in combination with the appropriate *cis* elements) and/or ADs from other transcription factors can be employed in a similar manner *(4,5)*.

A tremendously useful approach with the yeast two-hybrid is the searching of cDNA libraries for interacting partners of a protein of interest *(6)*. In this way, large numbers of proteins can be tested in pair-wise combinations with the protein of interest and those that interact can be selected for by GAL4-UAS sequences located upstream of reporter genes (*HIS3*, *ADE2*, etc.). In about 3–4 wk, a researcher can go from having a protein of interest a collection of candidate interacting partners that can be explored by further experimentation.

The interaction between two DNA-damage repair pathway components, Mec3p and Rad17p, will be used as an example for a yeast two-hybrid investigation of an interacting protein pair *(7)*. Additionally, the steps involved in carrying out a two-hybrid screen of an activation domain library for novel interacting partners will be described.

2. Materials

Unless otherwise noted, all chemicals are from Sigma.

1. Yeast Strain PJ694A: *Matα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ (8).*
2. Yeast Strain PJ694α: *Matα, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ (9).*
3. pOAD: GAL4 activation domain fusion vector with *LEU2* marker *(7,10)*.

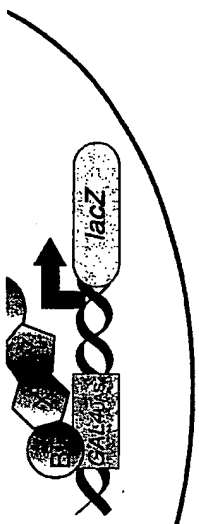

Fig. 1. *(opposite page)* The yeast two-hybrid system. A yeast cell expressing protein X fused to the DNA-binding domain (BD) of Gal4p does not activate reporter genes despite binding of BD-X to the $GAL4_{UAS}$ sequences upstream of the reporter genes (**A**). Similarly, expression of protein Y fused to the transcriptional activation domain (AD) in a yeast cell is not sufficient to stimulate transcription of the reporter genes (**B**). However, upon co-expression of the BD-X and AD-Y in the same cell by either co-transformation or mating, if X and Y interact, then the binding of the BD to the $GAL4_{UAS}$ sequence recruits the AD to the reporter gene promoter, which in turn recruits the transcriptional machinery and thus activates transcription of the reporter genes: *HIS3* encoding an enzyme involved in histidine biosynthesis; *ADE2* encoding an enzyme involved in adenine biosynthesis; and *lacZ*, the gene for the bacterial β-galactosidase enzyme (**C**).

4. pOBD: GAL4 DNA-binding domain fusion vector with *TRP1* marker *(7,10)*.
5. cDNA of gene of interest (or yeast genomic DNA).
6. Oligonucleotide primers.
7. Activation domain library from relevant organism/tissue source (store at –20°C).
8. YEPD medium: 1% yeast extract, 2% Bacto-peptone (Difco), 1.4% Meer agar (Difco). After autoclaving, add 2% dextrose (Fisher) (glucose)(4°C). Omit agar for liquid.
9. Synthetic dropout medium (SD): 0.67% yeast nitrogen base (Difco), 1.4% Meer agar. After autoclaving, add 1X amino acid mix and 2% glucose. Omit agar for liquid. Dropout medium has the above ingredients minus the appropriate supplement: No tryptophan (–Trp), no leucine (–Leu), no tryptophan/leucine (–Trp –Leu), no tryptophan/leucine/adenine (–Trp, –Leu, –Ade), or no tryptophan/leucine/histidine with added 3AT (–Trp, –Leu, –His + X m*M* 3AT) (4°C).
10. 3AT stock: Make 1 *M* 3-amino-1,2,4-triazole stock in ddH_2O (store at –20°C).
11. Yeast β-galactosidase Assay Kit (Pierce; cat. no. 75768) (–20°C) *(12)*.
12. *E. coli* strains DH5α and MH4 (*leuB*$^-$) prepared for electroporation *(17)*.
13. M9 Minimal medium plates (1 L): 750 mL ddH_2O, 5X M9 salts, 15 g Meer agar, water up to 980 mL; then 2 mL 1 *M* $MgSO_4$, 0.1 mL 1 *M* $CaCl_2$. After autoclaving, add 20 mL 20% glucose (4°C) *(17)*.
14. 5X M9 salts (1 L): 64 g $Na_2HPO_4{\cdot}7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5 g NH_4Cl *(17)*.
15. LB plates (1 L): 14 g Meer agar, 10 g tryptone, 5 g yeast extract, 5 g NaCl (Fisher), 1 mL 1 N NaOH. After autoclaving, add ampicillin (100 μg/mL: final concentration) (4°C).
16. PCR enzyme, restriction enzymes, and T4 DNA ligase.
17. Yeast 10X "Amino Acid" mix (per liter): 0.2 g adenine, 0.2 g arginine, 0.2 g histidine, 0.3 g isoleucine, 1 g leucine, 0.3 g lysine, 0.2 g methionine, 0.5 g phenylalanine, 2 g threonine, 0.2 g tryptophan, 0.3 g tyrosine, 0.2 g uracil, 1.5 g valine. Leave out appropriate components for dropout medium.
18. LiOAc-PEG solution: 40% PEG (Fisher), 100 m*M* lithium acetate (LiOAc), 10 m*M* Tris-HCl, 1 m*M* EDTA (TE). Make up fresh from 50% PEG 4000, 1 *M* LiOAc, and 1 *M* Tris-HCl, pH 7.5, 200 m*M* EDTA (TE). Vortex vigorously to get complete mixing.
19. Sheared Salmon Sperm DNA (Sigma) (5 mg/mL).
20. Qiagen Miniprep Kit and Qiagen Maxiprep Kit (Qiagen).
21. Qiagen Qiaquick Gel Extraction Kit (Qiagen).
22. 400–500 μm acid-washed glass beads (Sigma).
23. Replica stamp and velvets (wrapped in foil and autoclaved prior to use).

3. Methods

Described below are the (1) generation of the two-hybrid constructs, (2) assaying for an interaction, (3) screening of a library for the identification of new interacting partners, and (4) retesting of the newly identified protein partners.

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2. Insertion int TAGTAAC. **GCTGACC** with {5'-TC. GGTCGAC(

3.1.2. Cloning

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3. The superna DNA that ha each tube.
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5. Following v· were added vortexed.
6. 500 μL of f done by pip·
7. 57 μL of DN 30°C for 15

3.1. Building Two-Hybrid Constructs

3.1.1. Generating Inserts by PCR

Insertion of *MEC3* and *RAD17* into both of the pOAD and pOBD vectors was carried out by homologous recombination in yeast (*see* **Note 1**). The primer sequences below are from ref. *7*.

1. By the polymerase chain reaction (PCR), the sequence {5'-CTATCTATTCG ATGATGAAGATACCCCACCAAACCCAAAAAAAGAGATC**GAATTCCA GCTGACCACC**-3'} was added to the 5'-end of each gene for insertion into pOAD. The sequence {5'-CTTGCGGGGTTTTTCAGTATCTACGATTCATAG ATCTCTGCAGGTCGACGGATCCCCGGGAATTGCCATG-3'} was added to the 3' end of the genes.
2. Insertion into pOBD was mediated by the sequence {5'-ATCGGAAGAGAG TAGTAACAAAGGTCAAAGACAGTTGACTGTATCGCCG**GAATTCCA GCTGACCACC**-3'} added onto the 5' end, and extending the 3' end of the genes with {5'-TCATAAATCATAAGAAATTCGCCCGGAATTAGCTTGGCTGCA GGTCGACG**GATCCCCGGGAATTGCCATG**-3'}.

3.1.2. Cloning by Homologous Recombination in Yeast

The PCR products of *MEC3* and *RAD17* were co-transformed into yeast along with pOAD or pOBD vectors that had been cut with the *Nco*I restriction enzyme and gel-purified. As a control, vector alone was also transformed to identify the amount of uncut plasmid present. Transformation was carried out by the lithium acetate/polyethelene glycol method as follows (modified from *11*):

1. A single yeast colony of strain PJ694A (for pOAD) or PJ694α for (pOBD) was inoculated into 5 mL of liquid YEPD and grown overnight at 30°C with shaking.
2. One milliliter of the saturated yeast culture was then pelleted in an Eppendorf tube for each transformation to be performed (three for each strain, i.e., *RAD17*, *MEC3*, and no insert).
3. The supernatant was aspirated off and 3 µL of sheared 5 mg/mL salmon sperm DNA that had been boiled 5 min and transferred immediately to ice was added to each tube.
4. Tubes were vortexed and 1 µL of gel-extracted, *Nco*I-cut pOAD was added to each tube of PJ694A, while 1 µL of gel-extracted, *Nco*I-cut pOBD was added to each tube of PJ694α.
5. Following vortexing, 5 µL of the appropriate PCR products of *MEC3* and *RAD17* were added to a single tube of the appropriate strain and all tubes were again vortexed.
6. 500 µL of freshly made LiOAc/PEG was added to each tube and mixing was done by pipetting the viscous solution up and down several times.
7. 57 µL of DMSO was then added, the tubes were vortexed, and then incubated at 30°C for 15 min.

8. Following this incubation, the transformations were heat-shocked by a 15-min incubation in a 42°C water bath.
9. The cells were collected by a 1-min centrifugation in a microfuge, and the supernatant was aspirated off. 500 µL of 1X TE was used to resuspend the cells, followed by an additional 1-min centrifugation.
10. 400 µL of the supernatant was removed, and the cells were resuspended in the remaining approx 100 µL and plated onto SD plates lacking the appropriate amino acid (–Leu for pOAD constructs, –Trp for pOBD). The plates were incubated upside down for 3 d at 30°C.

3.1.3. Confirming Insertion of the Gene into the Vector

A comparison of the number of colonies on plates where PCR product was added versus the vector alone control gives an estimate of the number of colonies that contain insert.

1. Five colonies of each of the BD fusions and each of the AD fusions of *MEC3* and *RAD17* were grown in 5 mL of the appropriate SD medium for plasmid isolation (*see* **Subheading 3.4.1.**).
2. The plasmids were electroporated into *E. coli* (DH5α), isolated from the overnight *E. coli* culture using the Qiagen Miniprep Kit, and sequenced.
3. In-frame fusions of each gene with the AD or BD were used for the two-hybrid assay (*see* **Note 2**).

3.2. Performing the Assay

3.2.1. Mating of Yeast Cells Expressing Hybrids

Individual colonies of each haploid (PJ694A) expressing an AD fusion were resuspended in a small volume of water and then 2 µL of each cell suspension was spotted onto a YEPD plate. Onto these spots the appropriate BD fusion yeast (PJ694α) suspensions were spotted and the plates were incubated for 24 h at 30°C.

The YEPD plates were then replica-plated using an autoclaved velvet onto diploid selective plates, i.e., SD/ –Trp –Leu. This plate selects for diploids because only the yeast cells containing both the *LEU2*-bearing pOAD and *TRP1*-carrying pOBD will be able to grow: the only cells with both plasmids will be those that have mated (PJ694A/α diploids). The diploid plates were grown for 3 d at 30°C.

3.2.2. Replica Plating to Change to Selective Media

Diploids were then replica-plated using a stamp and autoclaved velvet onto plates to assess reporter gene activation. These were (SD/ –Trp –Leu) –Ade, –His, and –His with 3AT (3 m*M*, 30 m*M*, 50 m*M*, and 100 m*M*, respectively) (*see* **Note 3**). Plates were allowed to grow for 3 d at 30°C (**Fig. 2**).

pOBD

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BDRad17

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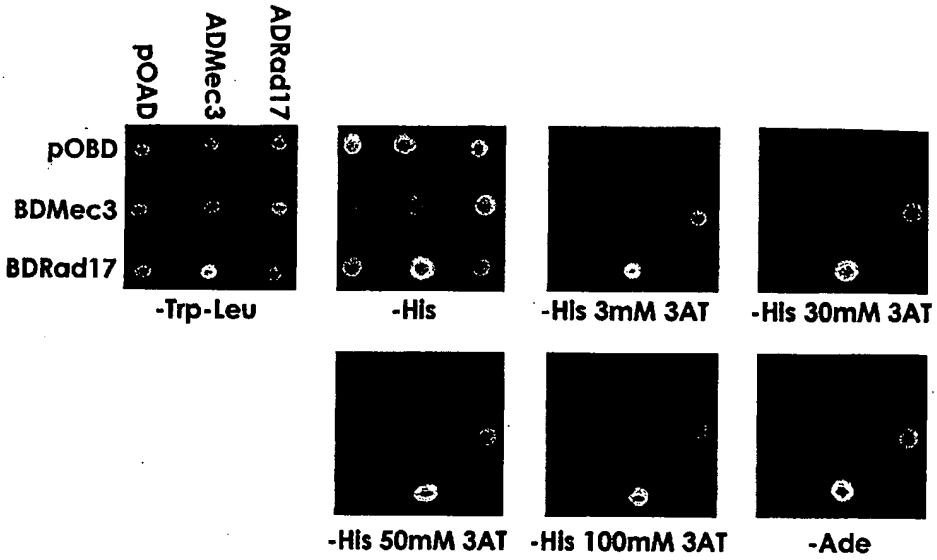

Fig. 2. The two-hybrid interaction of Mec3p and Rad17p demonstrated on selective plates. Each row contains a different BD construct and each column a different AD construct. Diploids containing the different AD and BD pairs were spotted onto SD plates lacking the indicated components and containing the indicated concentration of 3AT. Plates were incubated for 3 d at 30°C. Note that yeast containing the empty vector controls are able to grow on the SD/–His plate (*see* **Note 3**).

3.2.3. Assessing the Strength of Reporter Gene Transcription by a β-Galactosidase Assay

The Yeast β-Galactosidase Assay Kit from Pierce was used to carry out β-galactosidase assays on each of the diploids. The protocol that accompanies the kit *(12)* was followed (**Fig. 3**).

3.3. Screening an Activation Domain Library for Interactions

Most often researchers do not have a specific pair of proteins they are assaying for an interaction. In addition, if a researcher is interested in a small number of potentially interacting proteins, it is often desirable to examine the proposed interactions by a more direct biochemical technique. However, when an investigator has a favorite protein and wants to find out what other proteins it interacts with, the two-hybrid system is one of the best ways to start searching for these other proteins. To do this, they make use of a library of fusions to the Gal4p-AD and search with their protein of interest as a fusion to the Gal4p-BD. In this way it is possible to perform one experiment and identify many interacting partners for a particular protein.

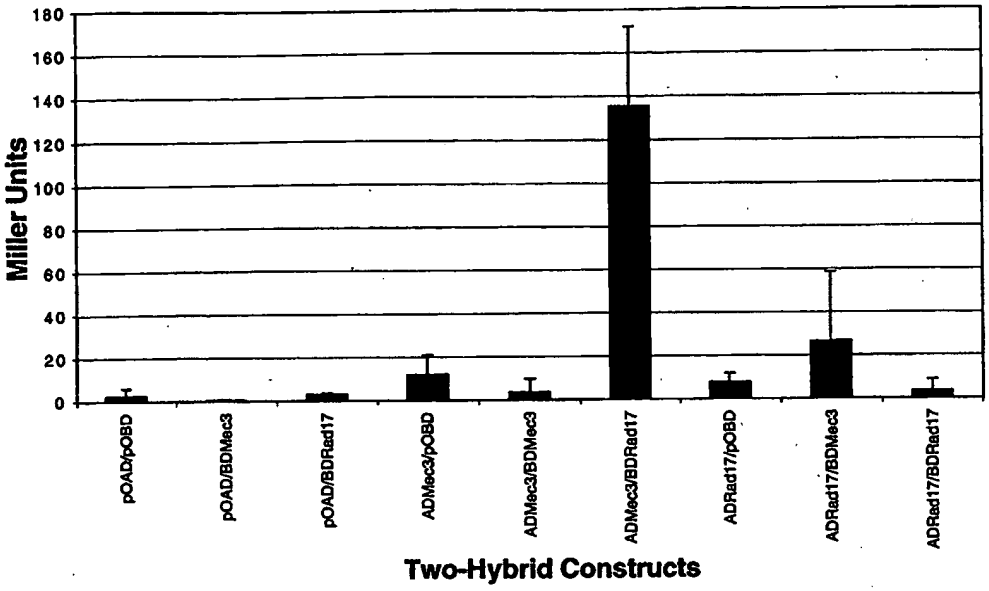

Fig. 3. β-galactosidase assays of Mec3p and Rad17p two-hybrid pairs. β-galactosidase activity was assayed using the Yeast β-Galactosidase Assay Kit (Pierce) *(12)*. Three replicates of each experiment were carried out.

A critical test prior to screening a library is to make sure that your protein fused to the Gal4p-BD does not activate the reporter genes on its own:

1. Co-transform yeast with pOAD and the BD-fusion of your protein of interest in pOBD.
2. Streak these transformants onto SD/–Trp –Leu –His +3m*M* 3AT plates and look for growth.
3. Do a β-galactosidase assay comparing your BD-fusion/pOAD to cells containing pOBD/pOAD.

If your fusion is expressed in yeast and does not self-activate, then you are ready to search an AD library for interacting partners. If the reporter genes are activated by your BD-fusion alone, then you may be able to overcome this with higher concentrations of 3AT or by using less than the full-length protein as a BD-fusion (*see* **Note 4**).

3.3.1. Getting a Good Library

The best way to find an activation domain library that will work for you is to search the literature. Look for a library that's reported in multiple publica-

tions and pay attention to the quantity and quality of the identified proteins found: Do screens with this library provide multiple clones encoding the same protein? Are the identified clones full-length cDNAs or only fragments? The answer to this latter question shouldn't deter you from obtaining and using libraries with fragments in them as these are sometimes superior to full-length clones in that interactions are found in the two-hybrid with fragments, but testing of the full-length protein diminishes or eliminates the strength of the interaction. These of course must be scrutinized with further experiments to confirm the authenticity of the interaction, but examples of such results have been reported *(13)*.

Additionally, the activation domain library should of course be from your organism of study if at all possible, and from the specific tissue(s) that are known to express your protein of interest. If these are not available, orthologues from a different organism can be tried against a library for the orthologous species. If something interesting is found, you can then go back to the original model organism and see if the proteins are interacting by two-hybrid or biochemical means in that system.

If no suitable libraries are available, you may wish to construct your own activation domain library either by doing it yourself or by paying to have one made [BD Biosciences Clontech offers this service *(14)*]. These alternatives offer you the ability to have the exact library you want, but at the expense associated with creating it. One caveat is that such a library will have been untested prior to your use, and its quality cannot be established to the same degree as one used by multiple other users who've already published interacting proteins found in the library.

As this is probably your first foray into the two-hybrid system, we will not describe the undertaking of building your own activation domain library, but other sources can provide you with the methodologies involved *(15)*. Instead, we will focus on screening the library with your protein fused to the Gal4p-BD. A flow-chart of library screening is included for clarity (**Fig. 4**).

3.3.2. Protocol for Screening

Depending on the form and amount of the library that you receive, you may have to amplify the library. Amplification is not desirable if it can be avoided because of the potential to lose rare clones in the process of growing up the library in *E. coli*. However, it is necessary if you are going to use the library for multiple screens. A good approximation is to use 50 µg of library DNA to transform a liter of yeast. More or less can be used, but should be based on the complexity of the library, i.e., how many different inserts are contained in the library.

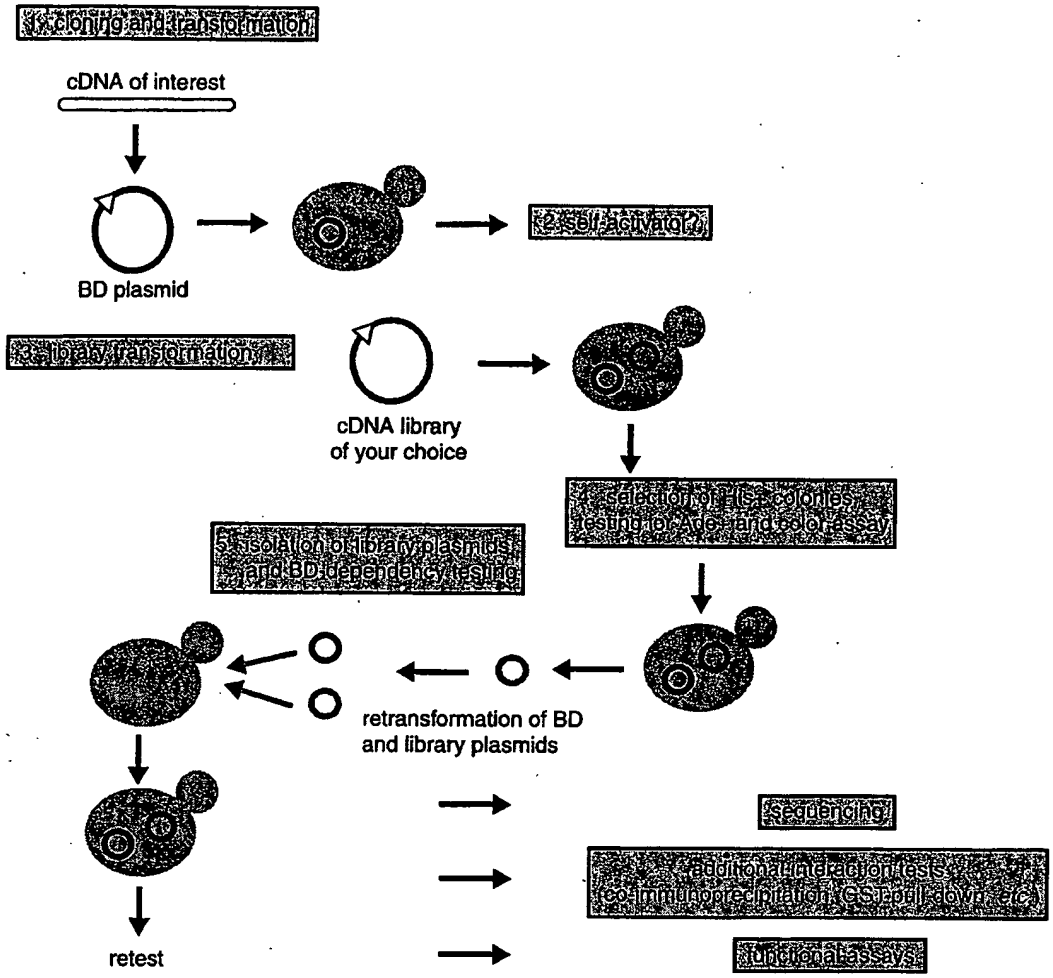

Fig. 4. Flow-chart for a yeast two-hybrid library screening. (1) The BD-fusion is transformed into yeast and (2) tested for reporter gene activation. (3) The AD-fusion library is transformed into cells bearing the BD-fusion and (4) the *HIS3* reporter gene is used to select for interaction positives. The other reporters are tested, and (5) plasmids are isolated and reintroduced into naive yeast cells for retesting of the reporter genes. Positive AD-fusions passing the retests are sequenced and further characterized.

You will want to obtain a multiple of transformants greater than this complexity (at least three times greater). For example, if the AD library you are using has 1×10^5 different inserts, if you get a million transformants (which should be possible from 50 μg of DNA and 1 L of yeast), you will have covered the library 10 times, or each insert is expected to be present in your transformation in 10 different colonies.

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3.3.2.1. Amplifying the Library

1. If it has not been provided to you, then determine the number of colony forming units/microgram (cfu/μg) of DNA by electroporating *E. coli* (DH5α) with a small fraction of the library DNA (e.g., 10 ng) and plating the transformants onto multiple 100 mm Petri plates with LB plus ampicillin. Also make 1:10, 1:100, and 1:1000 dilutions and plate these to ensure an accurate count of the cfu/μg. Based on this number, you can decide how many 150 mm LB amp plates you will need in order to plate out individual colonies (approx 2000 colonies per plate) for the actual amplification in the next step.
2. Electroporate *E. coli* (Dh5α) with the amount of the library determined in **step 1** and grow the plates overnight at 37°C.
3. Scrape the cells off of the plates with 1 mL of LB per plate and a spreader. [We have also used cell culture spatulas (costar® Cell Lifter, cat. no. 3008) for this purpose, but care must be taken not to dig up too much agar from the plate.]
4. Weigh the cell pellet and perform a MaxiPrep (Qiagen) using volumes based on this weight to isolate the DNA.
5. Use a fluorimeter and/or spectrophotometer to measure the concentration of DNA.

3.3.2.2. Transforming Yeast (Adapted from refs. *14* and *16*)

1. Grow 1 L of PJ694α previously transformed with your bait protein fused to the BD in YEPD to mid-log phase (1×10^7 cells/mL or an absorbance of approx 0.5–0.6 at a wavelength of 660 nm).
2. Pellet the cells in two 500 mL centrifuge tubes by centrifugation at 1000*g* for 15 min.
3. Resuspend the cells in 500 mL of ddH_2O. Repeat centrifugation.
4. Resuspend the cells in 8 mL of TE/LiOAc and transfer to a 200 mL centrifuge tube with screw cap.
5. Prepare 100 mL PEG/LiOAc being sure to vortex thoroughly as PEG is extremely viscous.
6. Add 20 mg of sheared salmon sperm DNA (boiled for 5 min then placed immediately on ice). Mix by vortexing.
7. Add 50–500 μg of the AD library DNA to the yeast. Mix by vortexing.
8. Add 60 mL of PEG/LiOAc and vortex to mix.
9. Shake the tube at 200 rpm at 30°C for 30 min.
10. Add DMSO to a final concentration of 10% and mix by inverting.
11. Heat shock the cells in a 42°C water bath for 15 min with occasional swirling.
12. Chill cells on ice.
13. Collect cells by centrifugation in swinging bucket rotor for 5 min at 1000*g*.
14. Resuspend the transformed cells in 10 mL of TE.
15. Plate out 100 μL of a 1:10, 1:100, and 1:1000 dilutions onto SD/–Trp, –Leu plates to determine the transformation efficiency.

16. Plate the remaining volume onto 40 (250 µL/ plate) 150 mm petri plates with SD/–Trp –Leu –His + 3 m*M* 3AT (or the 3AT concentration you've determined for your BD-fusion) (*see* **Note 4**).
17. Incubate plates upside down at 30°C for up to 2 wk (*see* **Note 5**). Check for positives after 3 d.

3.3.3. What Positives Should be Further Pursued?

When positive colonies have grown on the plates, streak them onto a new plate containing SD/–Trp –Leu –His + 3 m*M* 3AT. Anything that doesn't grow anew should be discarded. You can then check the other reporter genes by replica plating onto SD/–Trp –Leu –Ade, and carrying out β-galactosidase assays (*see* **Note 6**). Anything that isn't activating all three reporter genes should most likely be excluded.

The –Ade plate will most likely give you several variations in your positives: (i) white, strong growth; (ii) pink, moderate growth; and (iii) red, little or no growth. Pursue the white positives first, because they represent the strongest positives. However, the *ADE2* reporter is the most stringent and weak interactions that are "real" by the *HIS3* and *lacZ* reporters might be worth pursuing if you don't have many positives or none are white on a –Ade plate.

3.4. Retesting Interaction Positives

With a narrowed-down number of candidates to study further, you can begin the retesting process. You'll first want to ensure that the reporter gene activation is due to the two-hybrid plasmids by isolating the plasmids and moving them into naive yeast. It is important to then test the AD-fusions against the empty pOBD vector as well as other BD-fusions to determine if your positives activate reporter genes nonspecifically with any BD-fusion. Lastly, interactions observed by the two-hybrid should be reproduced by testing the reciprocal fusions and other experiments.

3.4.1. Isolating Plasmids for Retesting

1. Inoculate 5 mL of SD/ –Trp –Leu liquid media with a positive from your screen that has shown activation of all of the reporter genes. Grow overnight at 30°C with shaking.
2. Centrifuge to collect cells in a swinging bucket rotor for 5 min at 1000*g*.
3. Resuspend the pellet in 1 mL of water and transfer to an Eppendorf tube. Collect cells with a quick spin and aspirate off the supernatant.
4. Follow the Qiagen Miniprep protocol for bacterial plasmid isolation except upon addition of 250 µL of P1 buffer, also add approx 100 µL of 400–500 µm acid-washed glass beads. Vortex for 7 min. Continue with protocol as normal.
5. Electroporate *E. coli* strain MH4 (*leuB*⁻) prepared as described in *(17)* with 1.5 µL of the isolated DNA.

6. Plate half of the cells onto LB +ampicillin, and the other half onto an M9 minimal plate (*see* **Note 7**).
7. If using LB + ampicillin colonies, replica-plate or streak to M9 plates to select for bacteria containing the *LEU2* (AD-fusion) plasmids.
8. Miniprep the plasmid DNA and identify inserts by sequencing with primers located in the AD vector. We use the oligo [5'- TACCACTACAATGGAT GATGTATATAAC] to sequence the AD-protein junction, and the oligo [5'-GATGCACAGTTGAAGTGAACTTGCGG] to sequence the 3' end of the gene.
9. AD-fusions with in-frame inserts should be retested by assaying for an interaction with the BD-fusion used to first identify them by transforming (PJ694A) as in **Subheading 3.1.2.** and performing the two-hybrid as in **Subheading 3.2.** (*see* **Note 1**).

3.4.2. Testing Activation Domain Fusions Against Lamin

An important caveat for any positive found with a single bait plasmid is that the positive AD-fusion might be a false-positive. What is a "false" positive? The simple answer is that it is an AD-fusion that activates the reporter genes when combined with any (or at least a large number of) BD-fusions tested. The possible mechanisms for false-activation are complicated and not of interest to the researcher trying to identify a new interaction. So, how can a false-positive be identified? By testing multiple BD-fusions against it.

1. Transform PJ694A with the AD-fusion DNAs from **Subheading 3.4.1., step 9.**
2. Transform PJ694α with pOBD and at least one BD-fusion other than the one you did the screen with (BD-lamin is a good tester).
3. Follow the mating strategy for performing a two-hybrid as in **Subheading 3.2.** and examine the reporter genes for activation.
4. Discard AD-fusions that interact with unrelated BD-fusions as likely false-positives.
5. Study further those AD-fusions that are specific to your BD-fusion.

3.4.3. Independently Confirming Interactions

With interacting pairs of proteins in hand you'll want to move into your system to do experiments to confirm the importance of the interactions for your organism. This is the best way to confirm interactions, but depending on the difficulty and/or expense involved, it may be prudent to acquire independent confirmation of the interaction(s) you've found by other means. Biochemical methods for validating two-hybrid interactions are advised, including co-immunoprecipitation *(17)* and GST-pulldown *(17)*, both of which are described in this volume.

4. Notes

1. We make use of the ResGen (Invitrogen) yeast open reading frame (ORF) collection *(7)* in which every yeast ORF has the same 20 nucleotides on its 5' ends and a different 20 nucleotide sequence on its 3' end (shown in bold). These allow a single set of primers to be used to amplify any of the ORFs. Such a strategy expedites the construction of different fusions into the pOBD and pOAD vectors. However, if only a few two-hybrid constructs need to be made, cloning by restriction digests and ligation can be carried out using sites encoded in the ResGen 20mers that are contained head to tail in the vectors.
2. The commercially available antibodies we have tried that are raised against the Gal4p AD and BD don't work well to detect expression from the pOAD and pOBD vectors in our hands. Presumably this is due to the lower expression level of these CEN-based vectors relative to multicopy 2μ plasmids used for the two-hybrid in many other labs. If antibodies to the protein(s) of interest are available, then a Western blot would be another way to confirm in-frame insertion.
3. As shown in **Fig. 2**, PJ694A/α grow on –His alone due to leakiness of the *HIS3* reporter in these strains. To overcome this, 3-amino-1,2,4-triazole (3AT) is added to the medium because it is a stoichiometric inhibitor of the *HIS3* gene product. Thus, more 3AT included in the media means more transcription from the *HIS3* gene is required for growth. The strength of a two-hybrid signal can be qualitatively estimated from the concentrations of 3AT that yeast containing the two-hybrid pair is resistant to.
4. If your bait protein appears to be activating the reporter genes by itself, you can try growing the yeast with your BD-fusion on higher concentrations of 3AT until you find the point at which they are no longer resistant. Perform your screen at this concentration of 3AT. Alternatively, delete portions/domains of your protein with the purpose of removing the amino acids that are activating transcription and test the deletions for reporter gene activation prior to screening.
5. When storing plates for such a long time in the 30°C incubator, be sure to keep pans of clean water in the incubator to provide humidity. You can also incubate the plates in the plastic sleeves that the Petri plates came in to prevent excessive moisture loss.
6. A commonly used qualitative assay for β-galactosidase activity is the filter-lift. Unfortunately, this is not useful in the PJ694A/α strains because the strain itself is β-galactosidase positive by this assay. However, the quantitative liquid assay works well and the advantage of the three reporter genes outweighs the need for a quick β-galactosidase activity test present in other two-hybrid reporter strains.
7. The growth of the MH4 strain on the minimal plate will only be possible if it has been transformed with a *LEU2*-containing plasmid (the AD-fusion plasmid). Thus, you can separate the BD and AD plasmids by passaging through these *E. coli*. However, growth is slow such that 2 d are usually required to get colonies, so we also plate onto LB with ampicillin and after one day replica plate to M9 minimal medium to get good-sized colonies.

Acknowlec

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